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**ISOLATED PEPTIDES CORRESPONDING TO AMINO ACID  
SEQUENCES OF NY-ESO-1, <sup>W/58</sup>WHEREIN BIND TO MHC CLASS I  
AND MHC CLASS II MOLECULES, AND USES THEREOF**

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RELATED APPLICATIONS

This application is a continuation in part of Serial No. 09/062,422, filed April 17, 1998, <sup>now U.S. Patent No. 6,252,052,</sup> which is a continuation-in-part of Serial No. 08/937,263, filed September 15, 1997, <sup>now U.S. Patent No. 6,274,145,</sup> which is a continuation-in-part of Serial No. 08/725,182, filed October 3, 1996 <sup>now U.S. Patent No. 5,804,381</sup> <sup>and the issued U.S. Patent</sup> ~~now U.S. Patent No. 5,804,381~~. All of these applications are incorporated by reference.

FIELD OF THE INVENTION

This invention relates to HLA binding peptides derived from an antigen associated with cancer. These peptides bind to Class I and to Class II MHC molecules.

BACKGROUND AND PRIOR ART

It is fairly well established that many pathological conditions, such as infections, cancer, autoimmune disorders, etc., are characterized by the inappropriate expression of certain molecules. These molecules thus serve as "markers" for a particular pathological or abnormal condition. Apart from their use as diagnostic "targets", i.e., materials to be identified to diagnose these abnormal conditions, the molecules serve as reagents which can be used to generate diagnostic and/or therapeutic agents. A by no means limiting example of this is the use of cancer markers to produce antibodies specific to a particular marker. Yet another non-limiting example is the use of a peptide which complexes with an MHC molecule, to generate cytolytic T cells against abnormal cells.

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Preparation of such materials, of course, presupposes a source of the reagents used to generate these. Purification from cells is one laborious, far from sure method of doing so. Another preferred method is the isolation of nucleic acid molecules which encode a particular marker, followed by the use of the isolated encoding molecule to express the desired molecule.

To date, two strategies have been employed for the detection of such antigens, in e.g., human tumors. These will be referred to as the genetic approach and the biochemical approach. The genetic approach is exemplified by, e.g., dePlaen et al., Proc. Natl. Sci. USA 85: 2275 (1988), incorporated by reference. In this approach, several hundred pools of plasmids of a cDNA library obtained from a tumor are transfected into recipient cells, such as COS cells, or into antigen-negative variants of tumor cell lines which are tested for the expression of the specific antigen. The biochemical approach, exemplified by, e.g., O. Mandelboim, et al., Nature 369: 69 (1994) incorporated by reference, is based on acidic elution of peptides which have bound to MHC-class I molecules of tumor cells, followed by reversed-phase high performance liquid chromatography (HPLC). Antigenic peptides are identified after they bind to empty MHC-class I molecules of mutant cell lines, defective in antigen processing, and induce specific reactions with cytotoxic T-lymphocytes. These reactions include induction of CTL proliferation, TNF release, and lysis of target cells, measurable in an MTT assay, or a <sup>51</sup>Cr release assay.

These two approaches to the molecular definition of antigens have the following disadvantages: first, they are enormously cumbersome, time-consuming and expensive; and second, they depend on the establishment of cytotoxic T cell lines (CTLs) with predefined specificity.

The problems inherent to the two known approaches for the identification and molecular definition of antigens is best demonstrated by the fact that both methods have, so far, succeeded in defining only very few new antigens in human tumors. See, e.g., van der Bruggen et al., Science 254: 1643-1647 (1991); Brichard et al., J. Exp. Med. 178: 489-495 (1993); Coulie, et al., J. Exp. Med. 180: 35-42 (1994); Kawakami, et al., Proc. Natl. Acad. Sci. USA 91: 3515-3519 (1994).

Further, the methodologies described rely on the availability of established, permanent cell lines of the cancer type under consideration. It is very difficult to establish cell lines from certain cancer types, as is shown by, e.g., Oettgen, et al., Immunol. Allerg. Clin. North. Am. 10: 607-637 (1990). It is also known that some epithelial cell type cancers are poorly susceptible to CTLs in vitro, precluding routine analysis. These problems have stimulated the art to develop additional methodologies for identifying cancer associated antigens.

One key methodology is described by Sahin, et al., Proc. Natl. Acad. Sci. USA 92: 11810-11913 (1995), incorporated by reference. Also, see U.S. Patent No. 5,698,396, and Patent Application Serial No. 08/479,328 filed January 3, 1996. All three of these references are incorporated by reference. To

summarize, the method involves the expression of cDNA libraries in a prokaryotic host. (The libraries are secured from a tumor sample). The expressed libraries are then immnoscreened with absorbed and diluted sera, in order to detect those antigens which elicit high titer humoral responses. This methodology is known as the SEREX method ("Serological identification of antigens by Recombinant Expression Cloning"). The methodology has been employed to confirm expression of previously identified tumor associated antigens, as well as to detect new ones. See the above referenced patent applications and Sahin, et al., supra, as well as Crew, et al., EMBO J 144: 2333-2340 (1995).

The SEREX methodology has been applied to esophageal cancer samples, and an antigen has now been identified, and its encoding nucleic acid molecule isolated and cloned. This is the subject of several patent applications, some of which are incorporated by reference. The antigen and truncated forms have been found to be reactive with antibodies in the serum of cancer patients. It has also been found that peptides derived from this molecule bind with MHC molecules, provoking both cytolytic T cell and helper T cell responses. These features of the invention are seen in the disclosure which follows.

#### **BRIEF DESCRIPTION OF THE FIGURES**

Figure 1 shows the expression pattern of RNA for the NY-ESO-1 antigen, in various tissue types.

Figure 2 shows Northern Blot analysis of NY-ESO-1 mRNA, which was found in testis and cell line SK-MEL-19, but not in various other cell and tissue samples.

Figure 3 shows potential sites for modification of the deduced amino acid sequence of NY-ESO-1.

Figure 4 is a hydrophilicity plot of NY-ESO-1, showing hydrophilic domains in the amino terminus and a long, hydrophobic stretch close to the carboxyl end.

Figure 5 shows the results of CTL lysis studies using various cells which are HLA-A2 positive, NY-ESO-1 positive, positive for both, or positive for neither.

Figure 6 presents data establishing that HLA-A2 is the presenting molecule for presentation of SEQ ID NO: 1 derived peptides.

#### DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

##### Example 1

Total RNA was extracted from a snap frozen specimen of well to moderately differentiated squamous cell cancer of the esophagus, using well known methods. See, e.g., Chomzynski, J. Analyt. Biochem. 162: 156-159 (1987), for one such method. This RNA was used to prepare a cDNA library which was then transfected into  $\lambda$ ZAP phage vectors, in accordance with the

manufacturer's instructions. The  $\lambda$ ZAP library was then transfected into E. coli, yielding  $1.6 \times 10^6$  primary isolates.

The SEREX methodology of Sahin, et al., Proc. Natl. Acad. Sci. USA 92: 11810-11813 (1995), incorporated by reference, was then used. In brief, autologous serum was stripped of antibodies against molecules which are endogenous to E. coli by combining the serum with lysates of E. coli transfected with phage  $\lambda$ ZAP which did not contain the cDNA clones from the esophageal cancer cells.

The depleted serum was then diluted, and mixed with nitrocellulose membranes containing phage plaques. The plaques were incubated overnight, at room temperature. Washing followed, and then the filters were incubated with alkaline phosphatase conjugated goat anti human ~~Fcy~~<sup>Fcp</sup> secondary antibodies, and reactive phage plaques were visualized by incubating with 5-bromo-4-chloro-indolyl phosphate and nitroblue tetrazolium. A total of 13 positive clones were found.

#### Example 2

Following identification, the reactive clones were subcloned to monoclonality via dilution cloning and testing with human serum. These clones were then purified, excised in vitro, and converted into pBK-CMV plasmid forms, using the manufacturer's instructions. The inserted DNA was then evaluated using EcoRI-XbaI restriction mapping to determine different inserts. Eight different inserts were identified, ranging in size from about 500 to about 1.3 kilobase pairs. The clones were sequenced using an ABI PRISM automated sequencer.

Table 1 summarizes the results. One gene was represented by four overlapping clones, a second by three overlapping clones, and the remaining six by one clone only.

A homology search revealed that the clones referred to as NY-ESO-2, 3, 6, 7 were already known. See Elisei, et al., J. Endocrin. Invest. 16: 533-540 (1993); Spritz, et al., Nucl. Acids Res. 15: 10373-10391 (1987); Rabbits, et al., Nature Genetics 4: 175-180 (1993); Crozat, et al., Nature 363: 640-644 (1993); GenBank H18368 and D25606. Two of the clones (NY-ESO-3 and NY-ESO-6), have previously been shown to be expressed in various normal human tissues. No evidence of lineage restriction has been found. NY-ESO-6 (cDNA), appears to be the 3'-untranslated portion of the FUS/TLS gene. In experiments not reported here, sequencing and Southern Blot analysis of NY-ESO-6 showed no evidence of translocation or point mutations in the cancer. Four of the clones, i.e., NY-ESO-1, 4, 5 and 8 showed no strong homology to sequences in the databases examined, and were thus studied further.



Table 1. Genes isolated from esophageal cancer library by immunoscreening with autologous serum

5	GENE	CLONE#	Size	DNA databank	Comments
10	NY-ESO-1	E1-5b E1-114b E1-153c E1-50	679bp 614bp 670bp 679bp	No strong homology	expressed in testis and ovary
15	NY-ESO-2	E1-71a E1-140 E1-31	605bp 874bp 750bp	U1 small nuclear RNP 1 homolog	cloned by Ab screening (thyroiditis patient)
20	NY-ESO-3	E1-141b	517bp	Colon 3' direct MboI cDNA; Adult brain cDNA	(dbj D25606, gb H18638) unpublished
25	NY-ESO-4	E1A-10c	400bp	No strong homology	ubiquitous expression in normal tissues
30	NY-ESO-5	E1A-54	670bp	No strong homology	expressed in normal esophagus
35	NY-ESO-6	E1B-9b	-1.2kb	Human <i>fus</i> mRNA	translocated in liposarcoma t(12;16)
40	NY-ESO-7	E1B-20f	-1.0kb	human U1-70k sn RNP	different from NY-ESO-2 (embl HSU17052, gbM22636)
45	NY-ESO-8	E1B-20g	-1.3kb	No strong homology	ubiquitous expression in normal tissues

Example 3

Studies were carried out to evaluate mRNA expression of the NY-ESO 1, 4, 5 and 8 clones. To do this, specific oligonucleotide primers were designed for each sequence, such

that cDNA segments of 300-400 base pairs could be amplified, and so that the primer melting temperature would be in the range of 65-70°C. Reverse transcription-PCR was then carried out using commercially available materials and standard protocols. A variety of normal and tumor cell types were tested. The clones NY-ESO-4 and NY-ESO-8 were ubiquitous, and were not studied further. NY-ESO-5 showed high level expression in the original tumor, and in normal esophageal tissue, suggesting that it was a differentiation marker.

NY-ESO-1 was found to be expressed in tumor mRNA and in testis, but not normal colon, kidney, liver or brain tissue. This pattern of expression is consistent with other tumor rejection antigen precursors.

#### Example 4

The RT-PCR assay set forth supra was carried out for NY-ESO-1 over a much more complete set of normal and tumor tissues. Tables 2, 3 and 4 show these results. In brief, NY-ESO-1 was found to be highly expressed in normal testis and ovary cells. Small amounts of RT-PCR production were found in normal uterine myometrium, and not endometrium, but the positive showing was not consistent. Squamous epithelium of various cell types, including normal esophagus and skin, were also negative.

When tumors of unrelated cell lineage were tested, 2 of 11 melanomas cell lines showed strong expression, as did 16 of 67 melanoma specimens, 6 of 33 breast cancer specimens and 4 of 4

bladder cancer. There was sporadic expression in other tumor types.

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Table 2: mRNA distribution of NY-ESO-1 in normal tissues

	Tissue	mRNA	Tissue	mRNA
5	Esophagus	-	Adrenal	-
	Brain*	-	Pancreas	-
	Fetal Brain	-	Seminal Vesicle	-
	Heart	-	Placenta	-
	Lung	-	Thymus	-
10	Liver	-	Lymph node	-
	Spleen	-	Tonsil	-
	Kidney	-	PBL	-
	Stomach	-	PBL, activated#	-
	Small intestine	-	Melanocytes	-
15	Colon	-	Thyroid	-
	Rectum	-	Uterus	+/-**
	Breast	-	Testis	+
	Skin	-	Ovary	+

\*tissues from several parts tested with IL-2 and PHA

\*\*weakly positive in some specimens, negative by Northern blot

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Table 3: mRNA distribution of NY-ESO-1 in melanoma and breast cancer cell lines:

Cell line	NY-ESO-1 mRNA
MZ2-MEL3.1	-
MZ2-MEL2.2	-
SK-MEL-13	-
SK-MEL-19	+
SK-MEL-23	-
SK-MEL-29	-
SK-MEL-30	-
SK-MEL-31	-
SK-MEL-33	-
SK-MEL-37	+
SK-MEL-179	-
SK-BR-3	-
SK-BR-5	-
734B	-
MDA-MB-231	-

Table 4: NY-ESO-1 mRNA expression in various human tumors by RT-PCR

tumor type	mRNA(positive/total)	tumor type	mRNA(positive/total)
melanoma	25/77	ovarian cancer	2/8
breast cancer	17/43	thyroid cancer	2/5
prostate cancer	4/16	bladder cancer	9/13
colon cancer	0/16	Burkitt's lymphoma	1/2
glioma	0/15	basal cell carcinoma	0/2
gastric cancer	0/12	Jejomysarcoma	0/2
lung cancer	5/17	other sarcomas	0/2
renal cancer	0/10	pancreatic cancer	0/2
lymphoma*	0/10	seminoma	0/1
hepatoma	2/7	spinal cord tumor	0/1

\*non-Hodgkin's, non-Burkitt's types.

A further set of experiments were carried out to ascertain if the presence of anti NY-ESO-1 antibody in cancer patient sera could be determined via an ELISA.

To elaborate, recombinant NY-ESO-1 in a solution of coating buffer (15 mM Na<sub>2</sub>CO<sub>3</sub>, 30 mM NaHCO<sub>3</sub>, pH 9.6, 0.02% NaN<sub>3</sub>), at a concentration of 1 ug/ml, was adsorbed to microwell plates (10 ul of solution per well), and then kept overnight at 4°C. The plates were washed with phosphate buffered saline, and blocked, overnight, at 4°C, with 10 ul/well of 2% bovine serum albumin/phosphate buffered saline. After washing, 10 ul/well of diluted serum in 2% bovine serum albumin was added to the wells. Following two hours of incubation at room temperature, plates were washed, and 10 ul/well of goat anti-human IgG-alkaline phosphatase conjugates were added, at a 1:1500 dilution. This solution was incubated for one hour at room temperature, followed by washing and addition of a solution of substrate for the alkaline phosphatase (10 ul/well). After 25 minutes at room temperature, the wells were read with a fluorescence plate reader. The results are presented in the following table:

	Eso 1 +/-total tested	%
Cancer patients:		
melanoma	12/127	9.4
ovarian cancer	4/32	12.5
lung cancer	1/24	4.0
breast cancer	2/26	7.7
Blood donors	0/70	0

In order to determine whether there was a relationship between expression of mRNA for NY-ESO-1 in tumors, and antibody response to the NY-ESO-1 protein, data from sixty-two melanoma patients were compared. All patients whose serum was reactive with NY-ESO-1 protein (i.e., contained antibodies to NY-ESO-1), also had NY-ESO-1 positive tumors, while no patients with NY-ESO-1 negative tumors showed antibodies to NY-ESO-1 in their serum. There was a percentage of NY-ESO-1 positive patients who lacked the antibody. Given that about 20-40% of melanomas expressed NY-ESO-1, and only patients with NY-ESO-1 positive tumors have antibody, the data suggest a high percentage of patients with NY-ESO-1 positive tumors develops antibodies against the protein, thus suggesting a broad scale assay useful in diagnosis and responsiveness to treatment.

#### Example 5

Northern blot analysis was then carried out to investigate the size of the NY-ESO-1 transcript, and to confirm tissue expression patterns. The methodology of Ausubel, et al., Current Protocols In Molecular Biology (John Wiley & Sons, 1995) was used. To be specific, 20 ug of total RNA per lane were dissolved in a formamide and formaldehyde containing buffer, heated to 65°C, and then separated on a 1.2% agarose gel, with 3% formaldehyde, followed by transfer to nitrocellulose paper. Hybridization was then carried out using a <sup>32</sup>P labelled probe, followed by high stringency washing. The final wash was at 0.1xSSC, 0.1% SDS, 60°C, for 15 minutes.

RNA from testis, and a melanoma cell line (SK-MEL-19) which had been positive for NY-ESO-1 in the prior assays, showed an RNA transcript of about 0.8-0.9 kb. An esophageal carcinoma specimen showed a smear in the 0.4-0.9 kb range, reflecting partial degradation. RNA from additional tissues or cell lines tested showed no transcript.

To get cDNA encoding the full transcript, the esophageal cDNA library was rescreened, using plaque hybridization, and the original cDNA clone as the hybridization probe. When  $3 \times 10^5$  clones were screened, six positives were found. The three longest clones were sequenced. Analysis of open reading frames showed that all three contained the entire coding region, and 5'-untranslated regions of variable size. The longest clone, 755 base pairs in length, (excluding polyA), contains a 543 base pair coding region, together with 53 untranslated bases at the 5' end and 151 untranslated base pairs at the 3'-end. See SEQ ID NO: 1 (also, Figure 3).

The long ORF indicated that the deduced sequence of NY-ESO-1 protein is 180 amino acids. The single immunopositive clone contained a sequence encoding 173 of these. Deduced molecular mass is 17,995 daltons.

Analysis shows that there is an abundance of glycine residues in the N-terminal portion (30 of the first 80, 4 in the remaining 100). Hydrophilicity analysis indicated that there were hydrophilic antigenic sequences in the N-terminal half of the molecule, with alternating hydrophobic and hydrophilic sequences, ending with a long, C-terminal hydrophobic tail (amino acids 152-172), followed by a short hydrophilic tail.



This pattern suggests a transmembrane domain. There are several potential N-myristoylation sites, 3 phosphorylation sites, and no evidence of N-glycosylation sites.

#### Example 6

A melanoma cell line "NW-MEL-38" was established, in 1995, from a patient who suffered from malignant melanoma. Serum samples, peripheral blood lymphocytes, and tumor samples, were taken from the subject and frozen, until the work described herein was carried out. In anticipation of evaluating antitumor T cell response in this patient, the patient was HLA typed as HLA-A1 and HLA-A2.

To determine whether melanoma from this patient expressed NY-ESO-1, total RNA was isolated from both tumor samples and cell line NW-MEL-38, using standard techniques. Then, two micrograms of the total RNA, from each samples were subjected to cDNA synthesis, again using standard techniques.

The cDNA was then used in RT-PCR experiments, using the following primers:

5'-CACACAGGAT CCATGGATGC TGCAGATGCG G'-3' (SEQ ID NO: 2), and  
CACACAAAGC TTGGCTTAGC GCCTCTGCCC TG-3' (SEQ ID NO: 3)

These primers should amplify a segment of SEQ ID NO: 1 which spans nucleotides 271 to 599.

Amplification was carried out over 35 cycles, using an annealing temperature of 60°C. The PCR products were visualized via ethidium bromide staining, on a 1.5% agarose gel.

The results indicated that both the tumor and the cell line expressed SEQ ID NO: 1. The cell line and tumor samples were used in subsequent experiments.

#### Example 7

5 The isolated cDNA molecule, discussed supra, was then used to make recombinant protein. Specifically, the cDNA was PCR amplified, using standard techniques, and was then cloned into a commercially available plasmid vector, i.e., pQE9, which contains His tags. In work not elaborated upon herein, a second vector, pQE9K was also used. This differs from pQE9 in that  
10 kanamycin resistance is imparted by pQE9K, rather than ampicillin resistance.

The plasmid vector was transformed into E. coli strain XL1-Blue, and positive transformants were identified via restriction mapping and DNA sequencing. Production of recombinant protein was induced using isopropyl  $\beta$ -D-thiogalactoside, and the protein was purified on an  $\text{Ni}^{2+}$  ion chromatography column, following well known procedures. The protein when analyzed via 15% SDS-PAGE and silver staining, was identified as a protein with a molecular  
15 weight of about 22 kilodaltons. This is consistent with the anticipated size of the protein from its sequence. Two other forms of the recombinant protein were also identified. These consisted of amino acids 10-180, and 10-121 of the amino acid sequence reported in SEQ ID NO: 1. They have molecular weights  
20 of about 14 kD and 20 kD, respectively, on SDS-PAGE, as carried out supra.  
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5 An additional set of experiments were carried out to express NY-ESO-1 in baculovirus. To elaborate, the NY-ESO-1 cDNA insert was released from the pQE9 vector, by cleavage with BamHI and HindIII. This insert was then subcloned into a commercially available baculovirus vector which had been cleaved with the same enzymes. Positive clones were determined, using standard methods, and transfected into recipient Sf9 cells. Recombinant viruses were then used to infect insect cells, using a standard medium (IPL-41), supplemented with 10% fetal calf serum. The multiplicity of infection for the work was 20. Expression of recombinant protein was determined as described supra. The recombinant protein produced in this vector carries an His-tag, so it was purified on Ni<sup>2+</sup> affinity columns, also as described, supra. The protein consists of amino acids 10-180, and has a molecular weight of 20 kD via SDS-PAGE.

15 Additional eukaryotic transfectants were then produced. To do this, the NY-ESO-1 coding sequence was isolated from the pQE9 vector described supra, and then cloned into BamHI-HindIII sites of eukaryotic expression vector pcDNA 3.1. Next, COS-7 cells were transfected with this vector, by contacting cell samples with 150 ng of the plasmid discussed supra, and 150 ng of plasmid pcDNA 1 Amp, which contained either cDNA for HLA-A2.1 or cDNA for HLA-A1. The well known DEAE-dextran chloroquine method was used. The cells were then incubated at 37°C, for 48 hours, after which they were tested in a CTL stimulation assay. Specifically, the assay followed Traversari et al, Immunogenetics 35: 145-148 (1992), incorporated by reference. In brief, 2500 CTLs, (NW38-IVS-1, see example 9, infra), in 100

ul RPMI supplemented with 100% human serum, and 25 U/ml of recombinant IL-2 were added to microwells containing COS-7 transfectants (20,000 cells/well). After 24 hours, 50 ul of supernatant were collected from each well, and TNF- $\alpha$  levels were determined in a standard assay, i.e., one where cytotoxicity against WEHI 164 clone 13 cells were tested, using MTT. Positive cells were used in the Western Blot analysis, described in the example which follows.

The CTLs used were CTL NW38-IVS-1, prepared in accordance with Knuth et al., Proc. Natl. Acad. Sci. USA 81: 3511-3515 (1984), incorporated by reference. Specifically, mixed lymphocyte T cell cultures were set up, by combining  $10^5$  autologous NW38 MEL-1 tumor cells, and  $10^6$  peripheral blood lymphocytes, taken from the subject. The cytokine IL-2 was added, and the mixed culture was incubated for one week at 37°C. Tumor cells were removed, and a new aliquot of  $5 \times 10^4$  tumor cells were added together with IL-2. This process was repeated weekly, until a strong response was seen when tested against  $^{51}\text{Cr}$  labelled NW-MEL-38 cells. The responder T cells were collected and frozen until used in further experiments.

#### Example 8

Western Blot analysis was then carried out, using the serum samples described supra, as well as cell lysates taken from the cell line NW-MEL-38, described supra, and the COS-7 transfectants, described supra, and the purified recombinant protein, also described supra. Serum samples were taken from

various points of the patient's therapy. There was no difference in the results.

In these assays, 1 ug of recombinant NY-ESO-1 protein, or 5 ul of cell lysates of either type were diluted in SDS and boiled for five minutes, and then electrophoresed on a 15% SDS gel. After overnight blotting on nitrocellulose (0.45 um), and blocking with 3% BSA, the blots were incubated with serum, diluted at 1:1000, 1:10,000, and 1:100,000, or with a monoclonal antibody against NY-ESO-1, diluted to 1:50, as a positive control. The monoclonal antibody was prepared via Chen, et al., Proc. Natl. Acad. Sci. USA 5915-5919 (1996), incorporated by reference and elaborated as follows. BALB/C mice were immunized via five subcutaneous injections of recombinant NY-ESO-1 protein, at 2-3 week intervals. The immunizing formulation included 50 ug of recombinant protein in adjuvant. The first injection used Complete Freund's Adjuvant, and Incomplete Freund's Adjuvant was used thereafter. Spleen cells were taken from the immunized mice, and fused with mouse myeloma cell line SP2/0, to generate hybridomas. Representative hybridoma E978 was used for generation of mAbs.

Once hybridomas were generated, they were cloned, and their supernatants were screened against recombinant protein, using a standard solid phase ELISA on microtiter plates. The assay was in accordance with Dippold et al., Proc. Natl. Acad. Sci. USA 77: 6114-6118 (1980), incorporated by reference. A series of negative controls were also run, using recombinant NY-ESO-1. Serum antibodies which bound to recombinant protein, produced by E. coli as described, supra were visualized using goat anti-

human IgG, labelled with alkaline phosphatase at 1:10,000 dilution, and were then visualized with NBT-phosphate. Untransfected COS-7 cells were also used as a control. Serum from a healthy individual was also used as a control.

5 Strong reactivity against the recombinant protein was found at serum dilutions down to 1:100,000, and there was also reactivity against lysate of NW-MEL-38. There was no reactivity found against the untransfected COS-7 cells, nor did the serum from a healthy individual show reactivity.

10 Example 9

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15 Four different forms of NY-ESO-1 are described supra, i.e., the form produced by SEQ ID NO: 1 in E. coli, as well as one consisting of amino acids 10-180, one consisting of amino acids 10-121, and a form, expressed in the baculovirus vector system discussed supra which consisted of amino acids 10-180. Each form was used in ELISAs, following the above described protocols. All forms of the protein were found to be equally reactive with antibodies taken from various patients, as well as the murine monoclonal antibodies discussed, supra.

20 Example 10

25 In the testing of the COS-7 transfectants, supra, and the assays discussed in this example, a cytolytic T cell line "NW38-IVS-1" was used. This "CTL" was generated, via in vitro stimulation of the peripheral blood lymphocytes mentioned supra, using the tumor cell line NW-MEL-38. This was done using standard techniques.

The CTL was used in a cytotoxicity assay with NW-MEL-38 (which was HLA-A1, A2 positive, and NY-ESO-1 positive), along with two allogeneic cell lines which were NY-ESO-1 and HLA-A2 positive (SK-MEL-37 and MZ-MEL-19), a cell line which is MHC Class I negative (SK-MEL-19), a cell line which is HLA-A2 positive, but NY-ESO-1 negative (NW-MEL-145), along with control cell lines K562 and autologous phytohemagglutinin stimulated blasts. Various effector/target ratios were used, and lysis of <sup>51</sup>Cr labelled target cells was the parameter measured. Figure 5 shows this.

The results indicated that the CTL NW38-IVS-1 lysed both the autologous cell line NW MEL-38, and the allogeneic cell lines which were HLA-A2 and ESO-1 positive. Hence, the CTL was reactive with allogeneic materials. See figure 6.

#### Example 11

As patient NW38 was HLA-A1 and HLA-A2 positive, experiments were carried out to determine which MHC molecule was the presenting molecule.

The same experiment, described supra with COS-7 cells was carried out, except that, in these experiments, care was taken to secure separate groups of cotransformants which had been transformed with either HLA-A1 cDNA, or HLA-A2 cDNA, but not both. These results show that the CTL NW38-IVS-1 lysed COS-7 transfectants containing both NY-ESO-1 and HLA-A2 exclusively. See figure 6. The work also confirmed the specificity of the CTL, since the NY-ESO-1 negative, HLA-A2 positive cells

described in Example 9 were positive for other molecules known to be processed to peptides presented by HLA-A2 molecules.

#### Example 12

Once the presenting MHC molecule was identified as HLA-A2, a screening of the amino acid sequence for NY-ESO-1 was carried out, to identify all peptides which satisfy this motif, using the model set forth by D'Amaro et al., Human Immunol. 43: 13-18 (1995), and Drijfhout, et al., Human Immunol. 43: 1-12 (1995) incorporated by reference. Peptides corresponding to all of the amino acid sequences deduced thereby were synthesized, using standard techniques, and were then used in cytotoxicity assays, following Knuth et al., Proc. Natl. Acad. Sci. USA 81: 3511-3515 (1984), incorporated by reference. Specifically, cell line CEMX721.174.T2 ("T2" hereafter), was used, because it does not process antigens to MHC complexed peptides, thereby making it ideal for experiments of the type described herein. Samples of T2 cells were labelled with 100 uCi of Na(<sup>51</sup>Cr)O<sub>4</sub>, using standard methods, and were then washed three times, followed by incubation with 10 ug/ml peptide and 2.5 ug/ml of β2-microglobulin. Incubation was for one hour, at room temperature. Then responder cells (100 ul of a suspension of CTL NW38-IVS-1) were added, at an effector/target ratio of 90:1, and incubated for four hours in a water saturated atmosphere, with 5% CO<sub>2</sub>, at 37°C. Then, plates were centrifuged at 200xg for five minutes, 100 ul of supernatant was removed, and radioactivity was measured. The percentage of <sup>51</sup>Cr release was



determined in accordance with known strategies. it was found that the peptides SLLMWITQCFL (SEQ ID NO: 4), SLLMWITQC (SEQ ID NO: 5), and QLSLLMWIT (SEQ ID NO: 6), were the three best stimulators of CTLs. Comparable results were found when NW-MEL-38 and cell lines SK-MEL-37 and MZ-MEL-19 were used as targets, as is shown, supra.

### Example 13

The amino acid sequence of the protein encoded by SEQ ID NO: 1 was analyzed for peptide sequences which correspond to HLA binding motifs. This was done using the algorithm taught by Parker et al., J. Immunol. 142: 163 (1994), incorporated by reference. In the Table which follows, the amino acid sequence, the HLA molecule to which it presumably binds, and the positions in SEQ ID NO: 1 are given. The resulting complexes should provoke a cytolytic T cell response. This could be determined by one skilled in the art following methods taught by, e.g., van der Bruggen, et al., J. Eur. J. Immunol. 24: 3038-3043 (1994), incorporated by reference.

	Sequence	MHC/HLA Molecule	Positions
	GPESRLLEF	HLA-A1	82-90
	LLMWITQCF	HLA-A3	158-166
5	LMWITQCFL	HLA-A3	159-167
	EPTVSGNIL	HLA-A24	125-133
	LQLSISACL	HLA-A24	145-153
	GARGPESRL	HLA-B7	79-87
	APRGPHGGA	HLA-B7	60-68
10	ESRLLEFYL	HLA-B7	84-92
	APPLPVPVGV	HLA-B7	113-121
	FATPMEAEL	HLA-B7	96-104
	AADHRQLQL	HLA-B7	139-147
	GARGPESRL	HLA-B8	79-87
15	ESRLLEFYL	HLA-B8	84-92
	VPGVLLKEF	HLA-B35	118-126
	ESRLLEFYL	HLA-B35	84-92
	GARGPESRL	HLA-B35	79-87
	LEFYLAMPF	HLA-B44	88-96
20	PESRLLEFY	HLA-B44	83-91
	AELARRSLA	HLA-B44	102-110
	MEAEARRS	HLA-B44	100-108
	QQLSLLMWI	HLA-B52	154-162
	AQDAPPLPV	HLA-B52	110-118
25	LQLSISSCL	HLA-B52	145-153
	ITQCFLPVF	HLA-B52	162-170
	LLEFYLAMPF	HLA-A1	87-96
	GPESRLLEFY	HLA-A1	82-91
	PLPVPGVLLK	HLA-A3	115-124
30	RSLAQDAPPL	HLA-A24	107-116
	APPLPVPVGV	HLA-B7	113-122
	GARGPESRLL	HLA-B7	79-88
	GPHGGAASLG	HLA-B7	63-72
	APRGPHGGAA	HLA-B7	60-69
35	GPRGAGAARA	HLA-B7	44-53
	TAADHRQLQL	HLA-B8	138-147
	APPLPVPVGV	HLA-B52	113-122
	QQLSLLMWIT	HLA-B52	154-163
	LQQLSLLMWI	HLA-B52	153-162
40	KEFTVSNIL	HLA-B52	124-133

Example 14

Further experiments were carried out to identify additional relevant peptides. In these experiments, a stable tumor cell line, i.e., MZ-MEL-19 was used. This cell line had been established from a patient referred to as MZ19, using methods described by Jäger, et al., J. Exp. Med 187:265-269 (1998), incorporated by reference. A cytolytic T cell line, i.e., MZ2-MEL 19-IVS-1 was also established, using MZ-MEL-19 and the methods set forth in example 7, supra, as well as Jäger et al., supra. This CTL lysed the autologous tumor cell line, in an HLA-A2 restricted fashion. This was determined using standard methods. The model set forth by D'Amaro, et al., supra, was used to identify all sequences in ESO-1 which satisfy HLA-A2 binding motifs. Twenty six peptides were identified in this way. All were synthesized, purified, and tested for integrity, using standard methods. The peptides were then synthesized, and tested in the experiments which follow. MZ19-IVS-1 was used, together with the T2 cells described in Example 12. The peptides of SEQ ID NO: ID NOS.: 4 and 5 bound to HLA-A2 molecules, and were recognized by CTL MZ19-IVS-1, which lysed the cells. This CTL also recognized complexes of HLA-A2 and the decapeptide:

LLMWITQCFL

(SEQ ID NO.: 7), which is found at positions 156-167 of SEQ ID NO.: ID NO.1.

Example 15

a Further studies were <sup>carried out</sup> ~~characterized~~ to determine if CD4+ helper T cells recognized complexes of MHC-Class II molecules and peptides.

5 Tumor cell line MZ-MEL-19 has been typed as being HLA-DR53 positive. Hence, NY-ESO-1 was screened using Futaki, et al., Immunogenetics 42:299-301 (1995), incorporated by reference, which teaches binding motifs for HLA-DR53. A total of twenty eight peptides which, in theory, would bind to HLA-DR53, ~~and~~ ~~antigens presenting cells alone.~~

a  
a  
Peripheral blood lymphocytes ("PBLs"), were isolated from two patients with metastatic melanoma, who had been typed as HLA-DR53 positive.

15 The typing was performed using standard, commercially available reagents. One patient was typed as being positive for HLA-DRB1 (alleles 1501-05, 1601-1603, 1605 and 0701), HLA DRB4\* (alleles 0101-0103), and DRB5\* (alleles 0101), while the second patient was typed as positive for HLA-DRB1\* (alleles 1401, 1407, 1408, and 0901), HLA-DRB3\* (alleles 0201-0203), and DRB4\*  
20 (alleles 0101-0103). All alleles of HLA-DRB4\* are referred to as HLA-DR53, in accordance with Bodmer, et al., Human Immunol 34:4-18 (1992), incorporated by reference.

25 The PBLs were treated with magnetic beads coated with appropriate antibodies to deplete CD4+ and CD8+ T lymphocytes. The remaining cells were seeded in 24 well plates, at  $4 \times 10^6$  cells/well, and were allowed to adhere to the plastic of the wells for 24 hours. Any non-adhering cells were removed, and the remaining cells were used as antigen presenting cells.

These cells were stimulated with GM-CSF (1000 U/ml), and IL-4 (1000 U/ml) for 5 days, in 96-well, flat bottom nitrocellulose plates, which had been coated, overnight, at 4°C, with 5ug/ml of anti-gamma interferon antibodies. Cells were seeded at 3.5x10<sup>5</sup> cells/well.

The cells were then pulsed with 4ug/well of test peptide, or 2ug/well of the complete NY-ESO-1 protein, as a control.

Then CD4+ T cells were added (1x10<sup>5</sup>) cells/well, in RPMI 1640 medium augmented with 10% human serum, L-asparagine (50mg/l), L-arginine (242 mg/l), and L-glutamine (300 mg/l), together with 2.5 ng/ml of IL-2, to a final volume of 100 ul).

This mixture was incubated for 48 hours at 37°C in a water saturated atmosphere. Then, plates were washed, 6 times, with a solution of 0.05% Tween 20/PBS, and then biotinylated anti-interferon gamma antibody was added at 0.5 ug/ml. The antibody was incubated for 2 hours at 37°C, after which plates were developed with standard reagents, for 1 hour. Substrate 3-ethyl-9-amino carbazole was added, and incubated for 5 minutes, with positives being represented by red spots. The number of red spots/well was indicative of the frequency of CD4+ T lymphocytes which recognized complexes of peptide and HLA-DR53, or HLA-DR53 and a peptide processed from recombinant NY-ESO-1. As controls, assays were run using reagents alone (i.e., CD4+ cells alone, and the stain alone).

The following peptides were found to sensitize the CD4+ T lymphocytes to release gamma interferon.

AADHRQLQLSISSCLQQL

VLLKEFTVSGNILTIRLT

PLPVPGVLLKEFTVSGNI

(SEQ ID NOS.: 8-10)

5           These three peptides satisfy the motif for binding to HLA-DR53 set forth by Futaki, et al., supra, which is an anchor residue of Tyr, Phe, Trp, or Leu, followed by Ala or Ser three residues downstream.

          Additional peptides were found which bind to HLA-DR53.

10           These peptides are:

GAASGLNGCCRCGARGPE

SRLLFYLAMPFATPMEA

TVSGNILTIRLTAADHRQ

(SEQ ID NOS.: 11-13).

15           The foregoing examples describe the isolation of a nucleic acid molecule which encodes an esophageal cancer associated antigen. "Associated" is used herein because while it is clear that the relevant molecule was expressed by esophageal cancer, other cancers, such as melanoma, breast, prostate and lung also express the antigen.

20           The invention relates to those nucleic acid molecules which encode antigens as described, and which hybridize to reference sequence SEQ ID NO: 1 under stringent conditions. "Stringent conditions" as used herein refers to conditions such as those  
25           specified in U.S. Patent No. 5,342,774, i.e., 18 hours of hybridization at 65°C, followed by four one hour washes at 2xSSC, 0.1% SDS, and a final wash at 0.2xSSC, more preferably 0.1xSSC, 0.1% SDS for 30 minutes, as well as alternate conditions which

afford the same level of stringency, and more stringent conditions.

Also a part of the invention are expression vectors which incorporate the nucleic acid molecules of the invention, in operable linkage (i.e., "operably linked") to a promoter. Construction of such vectors is well within the skill of the art, as is the transformation or transfection of cells, to produce eukaryotic cell lines, or prokaryotic cell strains which encode the molecule of interest. Exemplary of the host cells which can be employed in this fashion are COS cells, CHO cells, yeast cells, insect cells (e.g., Spodoptera frugiperda), NIH 3T3 cells, and so forth. Prokaryotic cells, such as E. coli and other bacteria may also be used.

Also a part of the invention is the antigen described herein, both in original peptide form and in post translational modified form, as well as proteins consisting of at least amino acids 10-121, and no more than 10-180, of the protein encoded by SEQ ID NO: 1. The molecule is large enough to be antigenic without any posttranslational modification, and hence it is useful as an immunogen, when combined with an adjuvant (or without it), in both precursor and post-translationally modified forms. These proteins can be used to determine whether or not antibodies are present in a sample, such as serum or blood, as shown supra. The antibodies produced using this antigen, both poly and monoclonal, are also a part of the invention as well as hybridomas which make the monoclonal antibody. These can be used therapeutically or diagnostically as the whole molecule or in portions, as discussed infra. Also a part of the invention

are reactive fragments, such as Fab, F(ab)<sub>2</sub>' and other fragments, as well as chimeras, humanized antibodies, recombinantly produced antibodies, and so forth. Especially preferred are chimeras where the entire antibody but the complementarity determining regions <sup>i.e., the so-called</sup> "CDRS" <sup>are</sup> is human, but the CDRs are murine.

As is clear from the disclosure, one may use the proteins and nucleic acid molecules of the invention diagnostically. The SEREX methodology discussed herein is premised on an immune response to a pathology associated antigen. Hence, one may assay for the relevant pathology via, e.g., testing a body fluid sample of a subject, such as serum, for reactivity with the antigen per se. Reactivity would be deemed indicative of possible presence of the pathology so, too, could one assay for the expression of the antigen via any of the standard nucleic acid hybridization assays which are well known to the art, and need not be elaborated upon herein. One could assay for antibodies against the subject molecule, using standard immunoassays as well.

Analysis of SEQ ID NO: 1 will show that there are 5' and 3' non coding regions presented therein. The invention relates to those isolated nucleic acid molecules which contain at least the coding segment, i.e., nucleotides 54-593, and which may contain any or all of nucleotides 1-53 and/or 594-747 of SEQ ID NO: 1.

Further analysis, as discussed supra, reveals that the molecule is processed to peptides which provoke lysis by cytolytic T cells. Example 7 showed how this type of motif analysis can be carried out for HLA-A2 molecules. There has been a great deal of work in motifs for various MHC or HLA



5 molecules, which is applicable here. Hence, a further aspect of  
the invention is a therapeutic method, wherein one or more  
peptides which bind to an HLA molecule on the surface of a  
patient's tumor cells are administered to the patient, in an  
amount sufficient for the peptides to bind to the MHC/HLA  
10 molecules, and provoke lysis by T cells. The exemplification  
given supra for HLA-A2 molecules is by no means the only type of  
this administration that can be used. Any combination of  
peptides may be used, such as those for other HLA molecules,  
described supra. These peptides, which may be used alone or in  
combination, as well as the entire protein or immunoreactive  
portions thereof, may be administered to a subject in need  
thereof, using any of the standard types of administration, such  
as intravenous, intradermal, subcutaneous, oral, rectal, and  
15 transdermal administration. Standard pharmaceutical carriers,  
adjuvants, such as saponins, GM-CSF, and interleukins and so  
forth may also be used. Further, these peptides and proteins  
may be formulated into vaccines with the listed material, as may  
dendritic cells, or other cells which present relevant  
20 MHC/peptide complexes. These peptides may also be used to form  
multimeric complexes of HLA/peptides, such as those described by  
Dunbar, et al., Curr. Biol. 8: 413-416 (1998), incorporated by  
reference, wherein four peptide/MHC/biotin complexes are  
attached to a streptavidin or avidin molecule. Such complexes  
25 can be used to identify and/or to stimulate T cell precursors.

Similarly, the invention contemplates therapies wherein the  
nucleic acid molecule which encodes NY-ESO-1 is incorporated  
into a vector, such as an adenovirus based vector, to render it

transfectable into eukaryotic cells, such as human cells. Similarly, nucleic acid molecules which encode one or more of the peptides may be incorporated into these vectors, which are then the major constituent of nucleic acid bases therapies.

5 Any of these assays can also be used in progression/regression studies. One can monitor the course of abnormality involving expression of NY-ESO-1, simply by monitoring levels of the protein, its expression, and so forth using any or all of the methods set forth supra.

10 It should be clear that these methodologies may also be used to track the efficacy of a therapeutic regime. Essentially, one can take a baseline value for the NY-ESO-1 protein, using any of the assays discussed supra, administer a given therapeutic agent, and then monitor levels of the protein thereafter, observing changes in ESO-1 levels as indicia of the efficacy of the regime.

15 As was indicated supra, the invention involves, inter alia, the recognition of an "integrated" immune response to the NY-ESO molecule. One ramification of this is the ability to monitor the course of cancer therapy. In this method, which is a part of the invention, a subject in need of the therapy receives a vaccination of a type described herein. Such a vaccination results, e.g., in a T cell response against cells presenting HLA/peptide complexes on their cells. The response also  
20 includes an antibody response, possibly a result of the release of antibody provoking proteins via the lysis of cells by the T cells. Hence, one can monitor the effect of a vaccine, by monitoring an immune response. As is indicated, supra, an  
25

increase in antibody titer or T cell count may be taken as an indicia of progress with a vaccine, and vice versa. Hence, a further aspect of the invention is a method for monitoring efficacy of a vaccine, following administration thereof, by determining levels of antibodies in the subject which are specific for the vaccine itself, or a large molecules of which the vaccine is a part.

The effects of a vaccine can also be measured by monitoring the T cell-response of the subject receiving the vaccine. A number of assays can be used to measure the precursor frequency of these in vitro stimulated T cells. These include, but are not limited to, chromium release assays, TNF release assays, IFN $\gamma$  release assays, an ELISPOT assay, and so forth. Changes in precursor T cell frequencies can be measured and correlated to the efficacy of the vaccine. Additional methods which can be employed include the use of multimeric complexes of MHC/peptides. An example of such complexes is the tetrameric HLA/peptide-biotin-streptavidin system of Dunbar, et al. Curr. Biol. 8: 413-416 (1998), incorporated by reference.

The identification of NY-ESO-1 proteins as being implicated in pathological conditions such as cancer also suggests a number of therapeutic approaches in addition to those discussed supra. The experiments set forth supra establish that antibodies are produced in response to expression of the protein. Hence, a further embodiment of the invention is the treatment of conditions which are characterized by aberrant or abnormal levels of NY-ESO-1 proteins, via administration of antibodies, such as humanized antibodies, antibody fragments, and so forth.

These may be tagged or labelled with appropriate cystostatic or cytotoxic reagents.

T cells may also be administered. It is to be noted that the T cells may be elicited in vitro using immune responsive cells such as dendritic cells, lymphocytes, or any other immune responsive cells, and then reperfused into the subject being treated.

Note that the generation of T cells and/or antibodies can also be accomplished by administering cells, preferably treated to be rendered non-proliferative, which present relevant T cell or B cell epitopes for response, such as the epitopes discussed supra.

The therapeutic approaches may also include antisense therapies, wherein an antisense molecule, preferably from 10 to 100 nucleotides in length, is administered to the subject either "neat" or in a carrier, such as a liposome, to facilitate incorporation into a cell, followed by inhibition of expression of the protein. Such antisense sequences may also be incorporated into appropriate vaccines, such as in viral vectors (e.g., Vaccinia), bacterial constructs, such as variants of the known BCG vaccine, and so forth.

Also a part of the inventions are peptides, which can be nonamers, decamers, or undecamers defined as having a core sequence:

LLMWIT (SEQ ID NO: 14)

which have at least one additional residue terminal to the first L residue, preferably serine and may have as many as three, wherein Serine is linked to L to form -SL-, and 0-4 additional

amino acids at the C-terminus which, as shown supra, bind to HLA-A2 molecules, thereby provoking a CTL response. These peptides may be used therapeutically, via administration to a patient who is HLA-A2 positive, and expresses NY-ESO-1 in connection with a pathology, as well as diagnostically, i.e., to determine if HLA-A2 positive cells are present, or if relevant CTLs are present, and so forth.

The HLA-A2 molecule is an MHC Class I molecule, and T cells which respond to complexes of peptides and class I molecules are generally CD8<sup>+</sup> cells. Another subset of T cells, CD4<sup>+</sup> cells, responds to complexes of MHC-Class II molecules and peptides, and MHC-Class II restricted CD4<sup>+</sup> T cell responses against recombinant NY-ESO-1, presented by autologous cultured dendritic cells have been detected in melanoma patients. Specifically, in results not described herein, CD4<sup>+</sup> cells were separated from other cells from PBLs or serum samples, using well known techniques. Then, they were admixed with dendritic cells which had been pulsed with NY-ESO-1 protein. Proliferation of CD4<sup>+</sup> cells was observed, bringing another facet to the integrated immune response discussed herein. Hence, a further aspect of this invention are these CD4<sup>+</sup> T cells, peptides which bind to the MHC-Class II molecules, and their use in therapy.

Exemplary of the peptides defined by the core sequence of SEQ ID NO: 14 is the peptide defined by SEQ ID NO: 7. This peptide, as indicated, binds to HLA-A2 molecules. Hence it is a "marker" for HLA-A2, as well as a component of peptide/MHC complexes which stimulate proliferation of CTLs, as is described supra.

As the examples indicate, ESO-1 is also processed to peptides which complex to MHC Class II molecules, HLA-DR53 in particular. These molecules satisfy the motif described by Futaki, et al., supra, i.e., Tyr, Phe, Trp, or Leu, followed 3 amino acids later by Ala or Ser. Such peptides must be at least 18 amino acids long, and are preferably no more than 25 amino acids long. Preferably, they consist of 18 amino acids, such as SEQ ID NOS.: 8, 9, 10, 11, 12 and 13. These peptides serve to identify HLA-DR53 positive cells. Further, in the case of peptides such as SEQ ID NOS.: 8, 9 and 10, these can be used to provoke proliferation of helper T cells, as the examples show. All of the applications given supra for the MHC Class I restricted peptides are applicable to these Class II restricted peptides. Further, since the nature of the immune response is different with MHC-Class I/peptide complexes as compared to MHC-Class II/peptide complexes, one may combine both types of peptide, such as in immune compositions, thereby generating a combined immune response. Hence, all applications described can be used with just the Class I restricted peptides, with just the Class II restricted peptides, or with combinations of these.

Other features and applications of the invention will be clear to the skilled artisan, and need not be set forth herein.

The terms and expression which have been employed are used as terms of description and not of limitation, and there is no intention in the use of such terms and expression of excluding any equivalents of the features shown and described or portions thereof, it being recognized that various modifications are possible within the scope of the invention.